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(57) Abstract

The invention relates to a detection method performed on a maternal serum or plasma sample from a pregnant female, which method comprises detecting the presence of a nucleic acid of foetal origin in the sample. The invention enables non–invasive prenatal diagnosis including for example sex determination, blood typing and other genotyping, and detection of pre–eclampsia in the mother.

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NON-INVASIVE PRENATAL DIAGNOSIS

This invention relates to prenatal detection methods using
non-invasive techniques. In particular, it relates to prenatal diagnosis by
5   detecting foetal nucleic acids in serum or plasma from a maternal blood
sample.

Conventional prenatal screening methods for detecting foetal
abnormalities and for sex determination traditionally use foetal samples
derived by invasive techniques such as amniocentesis and chorionic villus
10   sampling. These techniques require careful handling and present a degree
of risk to the mother and to the pregnancy.

More recently, techniques have been devised for predicting
abnormalities in the foetus and possible complications in pregnancy, which
use maternal blood or serum samples. Three markers commonly used
15   include alpha-foetoprotein (AFP - of foetal origin), human chorionic
gonadotrophin (hCG) and estriol, for screening for Down's Syndrome and
neural tube defects. Maternal serum is also currently used for biochemical
screening for chromosomal aneuploidies and neural tube defects. The
passage of nucleated cells between the mother and foetus is now a well-
20   recognised phenomenon (Lo et al 1989; Lo et al 1996). The use of foetal
cells in maternal blood for non-invasive prenatal diagnosis (Simpson and
Elias 1993) avoids the risks associated with conventional invasive
techniques. WO 91/08304 describes prenatal genetic determination using
foetal DNA obtained from foetal cells in the maternal blood. Considerable
25   advances have been made in the enrichment and isolation of foetal cells
for analysis (Simpson and Elias 1993; Cheung et al 1996). However,
these techniques are time-consuming or require expensive equipment.

Recently, there has been interest in the use of plasma or
serum-derived DNA for molecular diagnosis (Mulcahy et al 1996). In
30   particular, it has been demonstrated that tumour DNA can be detected by

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the polymerase chain reaction (PCR) in the plasma or serum of some
patients (Chen *et al* 1996; Nawroz *et al* 1996).

GB 2 299 166 describes non-invasive cancer diagnosis by
detection of K-*ras* and N-*ras* gene mutations using PCR-based techniques.

5   It has now been discovered that foetal DNA is detectable in
maternal serum or plasma samples. This is a surprising and unexpected
finding; maternal plasma is the very material that is routinely discarded by
investigators studying non-invasive prenatal diagnosis using foetal cells in
maternal blood. The detection rate is much higher using serum or plasma
10   than using nucleated blood cell DNA extracted from a comparable volume
of whole blood, suggesting that there is enrichment of foetal DNA in
maternal plasma and serum. In fact, the concentration of foetal DNA in
maternal plasma expressed as a % of total DNA has been measured as
from 0.39% (the lowest concentration measured in early pregnancy), to as
15   high as 11.4% (in late pregnancy), compared to ratios of generally around
0.001% and up to only 0.025% for cellular fractions (Hamada *et al* 1993).
It is important that foetal DNA is found in maternal plasma as well as serum
because this indicates that the DNA is not an artefact of the clotting
process.

20   This invention provides a detection method performed on a
maternal serum or plasma sample from a pregnant female, which method
comprises detecting the presence of a nucleic acid of foetal origin in the
sample. The invention thus provides a method for prenatal diagnosis.

The term "prenatal diagnosis" as used herein covers
25   determination of any maternal or foetal condition or characteristic which is
related to either the foetal DNA itself or to the quantity or quality of the
foetal DNA in the maternal serum or plasma. Included are sex
determination, and detection of foetal abnormalities which may be for
example chromosomal aneuploidies or simple mutations. Also included is
30   detection and monitoring of pregnancy-associated conditions such as pre-

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eclampsia which result in higher or lower than normal amounts of foetal
DNA being present in the maternal serum or plasma. The nucleic acid
detected in the method according to the invention may be of a type other
than DNA e.g. mRNA.

5   The maternal serum or plasma sample is derived from the
maternal blood. As little as 10μl of serum or plasma can be used.
However it may be preferable to employ larger samples in order to
increase accuracy. The volume of the sample required may be dependent
upon the condition or characteristic being detected. In any case, the
10   volume of maternal blood which needs to be taken is small.

The preparation of serum or plasma from the maternal blood
sample is carried out by standard techniques. The serum or plasma is
normally then subjected to a nucleic acid extraction process. Suitable
methods include the methods described herein in the examples, and
15   variations of those methods. Possible alternatives include the controlled
heating method described by Frickhofen and Young (1991). Another
suitable serum and plasma extraction method is proteinase K treatment
followed by phenol/chloroform extraction. Serum and plasma nucleic acid
extraction methods allowing the purification of DNA or RNA from larger
20   volumes of maternal sample increase the amount of foetal nucleic acid
material for analysis and thus improve the accuracy. A sequence-based
enrichment method could also be used on the maternal serum or plasma to
specifically enrich for foetal nucleic acid sequences.

An amplification of foetal DNA sequences in the sample is
25   normally carried out. Standard nucleic acid amplification systems can be
used, including PCR, the ligase chain reaction, nucleic acid sequence
based amplification (NASBA), branched DNA methods, and so on.
Preferred amplification methods involve PCR.

The method according to the invention may be particularly
30   useful for sex determination which may be carried out by detecting the

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presence of a Y chromosome. It is demonstrated herein that using only 10μl of plasma or serum a detection rate of 80% for plasma and 70% for serum can be achieved. The use of less than 1ml of maternal plasma or serum has been shown to give a 100% accurate detection rate.

5   The method according to the invention can be applied to the detection of any paternally-inherited sequences which are not possessed by the mother and which may be for example genes which confer a disease phenotype in the foetus. Examples include:

a)   Foetal rhesus D status determination in rhesus negative mothers
10   (Lo *et al* 1993). This is possible because rhesus D positive individuals possess the rhesus D gene which is absent in rhesus D negative individuals. Therefore, the detection of rhesus D gene sequences in the plasma and serum of a rhesus D negative mother is indicative of the presence of a rhesus D positive foetus. This
15   approach may also be applied to the detection of foetal rhesus D mRNA in maternal plasma and serum.

b)   Haemoglobinopathies (Camaschella *et al* 1990). Over 450 different mutations in the beta-globin gene have been known to cause beta-thalassaemia. Provided that the father and mother carry different
20   mutations, the paternal mutation can be used as an amplification target on maternal plasma and serum, so as to assess the risk that the foetus may be affected.

c)   Paternally-inherited DNA polymorphisms or mutations. Paternally-inherited DNA polymorphisms or mutations present on either a Y or
25   a non-Y chromosome, can be detected in maternal plasma and serum to assess the risk of the foetus being affected by a particular disease by linkage analysis. Furthermore, this type of analysis can also be used to ascertain the presence of foetal nucleic acid in a particular maternal plasma or serum sample, prior to diagnostic
30   analysis such as sex determination. This application will require the prior genotyping of the father and mother using a panel of

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polymorphic markers and then an allele for detection will be chosen which is present in the father, but is absent in the mother.

The plasma or serum-based non-invasive prenatal diagnosis method according to the invention can be applied to screening for Down's
5 Syndrome and other chromosomal aneuploidies. Two possible ways in which this might be done are as follows:

a) It has been found that in pregnancy involving foetuses with chromosomal aneuploidies e.g. Down's Syndrome, the level of foetal cells circulating in maternal blood is higher than in
10 pregnancies involving normal foetuses (Bianchi et al 1996). Following the surprising discovery disclosed herein that foetal DNA is present in maternal plasma and serum, it has also been demonstrated that the level of foetal DNA in maternal plasma and serum is higher in pregnancies where the foetus has a
15 chromosomal aneuploidy than in normal pregnancies. Quantitative detection of foetal nucleic acid in the maternal plasma or serum e.g. a quantitative PCR assay, can be used to screen pregnant women for chromosomal aneuploidies.

b) A second method involves the quantitation of foetal DNA
20 markers on different chromosomes. For example, for a foetus affected by Down's Syndrome the absolute quantity of foetal chromosomal 21-derived DNA will always be greater than that from the other chromosomes. The recent development of very accurate quantitative PCR techniques, such as real time
25 quantitative PCR (Heid et al 1996) facilitates this type of analysis.

Another application of the accurate quantitation of foetal nucleic acid levels in the maternal serum or plasma is in the molecular monitoring of certain placental pathologies, such as pre-eclampsia. The
30 concentration of foetal DNA in maternal serum and plasma is elevated in

pre-eclampsia. This is probably due to the placental damage which occurs.

It is anticipated that it will be possible to incorporate the nucleic acid-based diagnosis methods described herein into existing prenatal screening programmes. Sex determination has successfully been performed on pregnancies from 7 to 40 weeks of gestation.

In the attached figures:

Figure 1 shows increased foetal DNA in aneuploid pregnancies compared to control pregnancies;

Figure 2 shows increased foetal DNA in pre-eclampsia compared to control pregnancies;

Figure 3 shows an amplification curve and threshold cycle for real time quantitative PCR;

Figure 4 shows foetal DNA concentrations in maternal samples for a number of subjects at different stages of gestation.

The invention will now be illustrated in the following Examples, which do not in any way limit the scope of the invention.

EXAMPLES

Example 1

Analysis of foetal DNA for sex determination

Patients

Pregnant women attending the Nuffield Department of Obstetrics & Gynaecology, John Radcliffe Hospital, Oxford were recruited prior to amniocentesis or delivery. Ethics approval of the project was obtained from the Central Oxfordshire Research Ethics Committee. Informed consent was sought in each case. Five to ten ml of maternal peripheral blood was collected into an EDTA and a plain tube. For women undergoing amniocentesis, maternal blood was always collected prior to

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the procedure and 10 ml of amniotic fluid was also collected for foetal sex determination. For women recruited just prior to delivery, foetal sex was noted at the time of delivery. Control blood samples were also obtained from 10 non-pregnant female subjects and further sample processing was

5 as for specimens obtained from pregnant individuals.

Sample preparation

Maternal blood samples were processed between 1 to 3 hours following venesection. Blood samples were centrifuged at 3000g and plasma and serum were carefully removed from the EDTA-containing

10 and plain tubes, respectively, and transferred into plain polypropylene tubes. Great care was taken to ensure that the buffy coat or the blood clot was undisturbed when plasma or serum samples, respectively, were removed. Following removal of the plasma samples, the red cell pellet and buffy coat were saved for DNA extraction using a Nucleon DNA extraction

15 kit (Scotlabs, Strathclyde, Scotland, U.K.). The plasma and serum samples were then subjected to a second centrifugation at 3000g and the recentrifuged plasma and serum samples were collected into fresh polypropylene tubes. The samples were stored at -20°C until further processing.

20 **DNA extraction from plasma and serum samples**

Plasma and serum samples were processed for PCR using a modification of the method of Emanuel and Pestka (1993). In brief, 200 μl of plasma or serum was put into a 0.5ml eppendorf tube. The sample was then heated at 99°C for 5 minutes on a heat block. The heated sample

25 was then centrifuged at maximum speed using a microcentrifuge. The clear supernatant was then collected and 10 μl was used for PCR.

DNA extraction from amniotic fluid

The amniotic fluid samples were processed for PCR using the method of Rebello *et al* (1991). One hundred μl of amniotic fluid was

30 transferred into a 0.5 ml eppendorf tube and mixed with an equal volume of

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10% Chelex-100 (Bio-Rad). Following the addition of 20 µl of mineral oil to

prevent evaporation, the tube was incubated at 56°C for 30 minutes on a

heat block. Then, the tube was vortexed briefly and incubated at 99°C for

20 minutes. The treated amniotic fluid was stored at 4°C until PCR and

5   10 µl was used in a 100µl reaction.

Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) was carried out

essentially as described (Saiki *et al* 1988) using reagents obtained from a

GeneAmp DNA Amplification Kit (Perkin Elmer, Foster City, CA, USA).

10   The detection of Y-specific foetal sequence from maternal plasma, serum

and cellular DNA was carried out as described using primers Y1.7 and

Y1.8, designed to amplify a single copy Y sequence (DYS14) (Lo *et al*

1990). The sequence of Y1.7 is 5' CAT CCA GAG CGT CCC TGG CTT 3'

[SEQ ID NO: 1] and that of Y1.8 is 5' CTT TCC ACA GCC ACA TTT GTC

15   3' [SEQ ID NO: 2]. The Y-specific product was 198 bp. Sixty cycles of Hot

Start PCR using Ampliwax technology were used on 10 µl of maternal

plasma or serum or 100 ng of maternal nucleated blood cell DNA

(denaturation step of 94°C 1 minute and a combined

reannealing/extension step of 57°C 1 minute). Forty cycles were used for

20   amplification of amniotic fluid. PCR products were analysed by agarose

gel electrophoresis and ethidium bromide staining. PCR results were

scored before the foetal sex was revealed to the investigator.

Results

25   Sensitivity of PCR assay

Serial dilutions of male genomic DNA in 1 µg of female

genomic DNA were performed and amplified by the Y-PCR system using

60 cycles of amplification. Positive signals were detected up to the

100,000 dilution, i.e., approximately the equivalent of a single male cell.

30   Amplification of foetal DNA sequence from maternal plasma and serum

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Maternal plasma and serum samples were collected from 43 pregnant women with gestational ages from 12 to 40 weeks. There were 30 male foetuses and 13 female foetuses. Of the 30 women bearing male foetuses, Y-positive signals were detected in 24 plasma samples and 21

5   serum samples, when 10 μl of the respective samples was used for PCR. When nucleated blood cell DNA was used for Y-PCR, positive signals were only detected in 5 of the 30 cases. None of the 13 women bearing female foetuses and none of the 10 non-pregnant female controls resulted in a positive Y signal when either plasma, serum or cellular DNA was amplified.

10   Accuracy of this technique, even with serum/plasma samples of only 10 μl, is thus very high and most importantly it is high enough to be useful. It will be evident that accuracy can be improved to 100% or close to 100%, for example by using a larger volume of serum or plasma.

15   **Example 2**

Quantitative analysis of foetal DNA in maternal serum in aneuploid pregnancies

The prenatal screening and diagnosis of foetal chromosomal aneuploidies is an important part of modern obstetrical care. Due to the

20   risks associated with invasive procedures such as amniocentesis and the impracticability of performing screening with invasive methods, much effort has been devoted to the development of non-invasive screening methods for foetal chromosomal aneuploidies. The two main non-invasive methods which have been developed are maternal serum biochemical screening

25   and ultrasound examination for nuchal translucency. These methods are both associated with significant false-positive and false-negative rates.

The demonstration of foetal nucleated cells in maternal circulation offers a new source of foetal material for the non-invasive diagnosis of foetal chromosomal aneuploidies (Simpson *et al* 1993). By

30   the use of foetal nucleated cell enrichment protocols, several groups have

reported the detection of aneuploid foetal nucleated cells isolated from
maternal blood (Elias *et al* 1992; Bianchi *et al* 1992). Recently, it has been
demonstrated that there is increased foetal nucleated cell number in
maternal circulation when the foetus is suffering from a chromosomal
5   aneuploidy (Bianchi *et al* 1997).

Patients samples

        Blood samples from pregnant women undergoing prenatal
testing were collected prior to any invasive procedure. The foetal
karyotype was confirmed by cytogenetic analysis of amniotic fluid or
10   chorionic villus samples. Approval was obtained from the Research Ethics
Committee of The Chinese University of Hong Kong. Blood samples were
collected into plain tubes. Following blood clotting, the samples were
centrifuged at 3000 g, and serum were carefully removed and transferred
into plain polypropylene tubes. The samples were stored at -70 °C or -20
15   °C until further processing.

DNA extraction from plasma and serum samples

        DNA from serum samples were extracted using a QIAamp
Blood Kit (Qiagen, Hilden, Germany) using the "blood and body fluid
protocol" as recommended by the manufacturer (Chen *et al* 1996). Four
20   hundred to 800 µl of plasma/serum sample was used for DNA extraction
per column. The exact amount used was documented to enable the
calculation of target DNA concentration.

Real time quantitative PCR

        Theoretical and practical aspects of real time quantitative
25   PCR were previously described by Heid *et al* (1996). Real time
quantitative PCR analysis was performed using a PE Applied Biosystems
7700 Sequence Detector (Foster City, CA, U.S.A.) which is essentially a
combined thermal cycler/fluorescence detector with the ability to monitor
the progress of individual PCR reactions optically. The amplification and
30   product reporting system used is based on the 5' nuclease assay (Holland

et al 1991) (the TaqMan assay as marketed by Perkin-Elmer). In this system, apart from the two amplification primers as in conventional PCR, a dual labeled fluorogenic hybridisation probe is also included (Lee *et al* 1993; Livak *et al* 1995). One fluorescent dye serves as a reporter (FAM,

5   i.e., 6-carboxyfluorescein) and its emission spectra is quenched by a second fluorescent dye (TAMRA, i.e., 6-carboxy-tetramethylrhodamine). During the extension phase of PCR, the 5' to 3'-exonuclease activity of the *Taq* DNA polymerase cleaves the reporter from the probe thus releasing it from the quencher, resulting in an increase in fluorescent emission at 518

10   nm. The PE Applied Biosystems 7700 Sequence Detector is able to measure the fluorescent spectra of the 96 amplification wells continuously during DNA amplification and the data are captured onto a Macintosh computer (Apple Computer, Cupertino, CA, U.S.A.).

The SRY TaqMan system consisted of the amplification

15   primers SRY-109F, 5'-TGG CGA TTA AGT CAA ATT CGC-3' [SEQ ID NO:3]; SRY-245R, 5'-CCC CCT AGT ACC CTG ACA ATG TAT T-3' [SEQ ID NO:4]; and a dual labeled fluorescent TaqMan probe SRY-142T, 5'-(FAM)AGC AGT AGA GCA GTC AGG GAG GCA GA(TAMRA)-3' [SEQ ID NO: 5]. Primer/probe combinations were designed using the Primer

20   Express software (Perkin-Elmer, Foster City, CA, U.S.A.). Sequence data for the SRY gene were obtained from the GenBank Sequence Database (accession number L08063).

TaqMan amplification reactions were set up in a reaction volume of 50 μl using components (except TaqMan probe and

25   amplification primers) supplied in a TaqMan PCR Core Reagent Kit (Perkin-Elmer, Foster City, CA, U.S.A.). The SRY TaqMan probe were custom-synthesised by PE Applied Biosystems. PCR primers were synthesised by Life Technologies (Gaithersburg, MD, U.S.A.). Each reaction contained 5 μl of 10X buffer A, 300 nM of each amplification

30   primers, 100 nM of the SRY TaqMan probe, 4 mM $MgCl_2$, 200 μM each of

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dATP, dCTP and dGTP, 400 µM dUTP, 1.25 units of AmpliTaq Gold and

0.5 unit AmpErase uracil N-glycosylase. Five to ten µl of the extracted

serum DNA was used for amplification. The exact amount used was

recorded for subsequent concentration calculation. DNA amplifications

5   were carried out in 96-well reaction plates that were frosted by the

manufacturer to prevent light reflection and were closed using caps

designed to prevent light scattering (Perkin-Elmer, Foster City, CA, U.S.A.).

Each sample was analysed in duplicate. A calibration curve was run in

parallel and in duplicate with each analysis. The conversion factor of 6.6

10   pg of DNA per cell was used for expressing the results as copy numbers.

Thermal cycling was initiated with a 2-minute incubation at 50

°C for the uracil N-glycosylase to act, followed by a first denaturation step

of 10 minutes at 95 °C. Then, 40 cycles of 95 °C for 15 s and 60°C for 1

minute were carried out.

15   Amplification data collected by the 7700 Sequence Detector

and stored in the Macintosh computer were then analysed using the

Sequence Detection System (SDS) software developed by PE Applied

Biosystems. The mean quantity of each duplicate was used for further

concentration calculation. The concentration expressed in copies/ml was

20   calculated using the following equation:

$$C = Q \times \frac{V_{DNA}}{V_{PCR}} \times \frac{1}{V_{ext}}$$

where   C = target concentration in plasma or serum (copies/ml);

Q = target quantity (copies) determined by sequence detector

25   in a PCR;

V_{DNA} = total volume of DNA obtained following extraction,

typically 50 µl per Qiagen extraction;

V_{PCR} = volume of DNA solution used for PCR, typically 5-10µl

V_{ext} = volume of plasma/serum extracted, typically 400-800µl

Anti-contamination measures

Strict precautions against PCR contamination were used (Kwok *et al* 1989). Aerosol-resistant pipette tips were used for all liquid
5 handling. Separate areas were used for the setting up of amplification reactions, the addition of DNA template and the carrying out of amplification reactions. The 7700 Sequence Detector offered an extra level of protection in that its optical detection system obviated the need to reopen the reaction tubes following the completion of the amplification
10 reactions, thus minimising the possibility of carryover contamination. In addition, the TaqMan assay also included a further level of anti-contamination measure in the form of pre-amplification treatment using uracil N-glycosylase which destroyed uracil containing PCR products (Longo *et al* 1990). Multiple negative water blanks were included in every
15 analysis.

Results

Development of real time quantitative PCR

To determine the dynamic range of real time quantitative
20 PCR, serial dilutions of male DNA were made in water consisting of the DNA equivalent from 1,000 cells to 1 cell and subjected to analysis by the SRY TaqMan system. The fewer the number of target molecules, the more amplification cycles were needed to produce a certain quantity of reporter molecules. The system was sensitive enough to detect the DNA
25 equivalent from a single target cell.

A parameter, termed the threshold cycle (C_T) could be defined which was set at 10 standard deviations above the mean base-line fluorescence calculated from cycles 1 to 15 and was proportional to the starting target copy number used for amplification (Heid *et al* 1996). A plot
30 of the threshold cycle (C_T) against the input target quantity, with the latter

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plotted on a common log scale, demonstrated the large dynamic range and accuracy of real time quantitative PCR.

The real time quantitative SRY system was insensitive to the existence of background female DNA from 0 to 12,800 female genome-
5   equivalents. This greatly simplified the application of this system as separate calibration curves did not have to be constructed for different cases due to the presence of different concentrations of foetal and maternal DNA.

Quantitative analysis of foetal SRY gene from maternal serum from
10   **aneuploid and control pregnancies**

Real time quantitative SRY PCR was carried out for serum DNA extracted from women bearing aneuploid and normal foetuses. Data from individual cases are plotted in Figure I. Foetal DNA concentration was higher in aneuploid than control pregnancies (Mann-Whitney U Test,
15   p=0.006).

Discussion

In this study we demonstrate that the concentration of foetal DNA in maternal serum is elevated in aneuploid pregnancies. These
20   results indicate that foetal DNA quantitation has the potential to be used as a new screening marker for foetal chromosomal aneuploidies. A large scale population-based study could be carried out to develop cutoff values for screening purposes. It would also be useful to investigate the correlation of foetal DNA concentration with the other biochemical markers
25   for maternal serum biochemical screening.

The mechanism(s) by which increased amounts of foetal DNA is liberated into maternal circulation in aneuploid pregnancies require further research. One possibility is related to the increased numbers of foetal nucleated cells which are released into the maternal blood in
30   aneuploid pregnancies (Bianchi *et al* 1997). Another possible mechanism

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may be increased cell death or turnover which may be associated with
chromosomal aneuploidies.

Example 3

5   **Non-invasive prenatal determination of foetal RhD status from plasma
of RhD-negative pregnant women**

Introduction

The rhesus blood group system is important in transfusion
10   and clinical medicine, being involved in haemolytic disease of the newborn,
transfusion reactions and autoimmune haemolytic anaemia. Despite the
widespread use of rhesus immunoglobulin prophylaxis in rhesus D (RhD)-
negative mothers, rhesus isoimmunisation still occurs. In those cases
where the father is heterozygous for RhD gene, there is a 50% chance that
15   the foetus is RhD-positive and 50% chance that the foetus is RhD-
negative. The prenatal determination of foetal RhD status in these cases
is clinically useful because no further prenatal invasive testing or
therapeutic manoeuvres are necessary if the foetus can be shown to be
RhD-negative.
20   Advances towards this goal have been made possible
recently through the cloning of the human RhD gene (Le Van Kim *et al*
1992) and the demonstration that RhD-negative individuals lack the RhD
gene (Colin *et al* 1991). Prenatal determination of foetal RhD status has
been performed using PCR-based techniques on amniotic fluid samples
25   (Bennett *et al* 1993).
            A number of groups have also investigated the possibility of
using foetal cells in maternal blood for the determination of foetal RhD
status (Lo *et al* 1993). The main problem with this approach is that the
system is not sufficiently reliable without foetal cell enrichment or isolation
30   procedure as demonstrated by the high false-positive and false-negative

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rates on unenriched samples. Foetal cell enrichment or isolation
procedures, on the other hand, are tedious and expensive to perform
(Geifman-Holtzman *et al* 1996; Sekizawa *et al* 1996).

Our discovery of the presence of foetal DNA in maternal
plasma and serum offers a new approach for non-invasive prenatal
diagnosis.

Materials and Methods
Patients

Pregnant women attending the Nuffield Department of
Obstetrics & Gynaecology were recruited with informed consent. Approval
of the project was obtained from the Central Oxfordshire Research Ethics
Committee. Women in the second trimester of pregnancy were recruited
just prior to amniocentesis. Blood samples were collected prior to any
invasive procedures. Ten ml of amniotic fluid was also collected for foetal
RhD genotyping. Women in the third trimester of pregnancy were recruited
just prior to delivery. A sample of cord blood was taken following delivery
for the ascertainment of foetal RhD status by serological methods.
Sample preparation

Blood samples were collected into tubes containing EDTA.
The samples were centrifuged at 3000 g, and plasma was carefully
removed and transferred into plain polypropylene tubes. Great care was
taken to ensure that the buffy coat was not disturbed. The buffy coat
samples were stored at -20 °C until further processing. The plasma
samples were then recentrifuged at 3000 g and plasma was again carefully
removed and transferred into a fresh set of plain polypropylene tubes. The
samples were stored at -20 °C until further processing.

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DNA extraction from plasma and serum samples

DNA from plasma and buffy coat samples were extracted using a QIAamp Blood Kit (Qiagen, Hilden, Germany) using the "blood and body fluid protocol" as recommended by the manufacturer (Cher *et al* 1996). Eight hundred μl of plasma sample and 200 μl of buffy coat sample was used for DNA extraction per column.

Real time quantitative PCR

Real time quantitative PCR analysis was performed as described in Example 2 with the following modifications.

The RhD TaqMan system consisted of the amplification primers RD-A: 5'-CCT CTC ACT GTT GCC TGC ATT-3' [SEQ ID NO: 6]; RD-B: 5'-AGT GCC TGC GCG AAC ATT-3' [SEQ ID NO: 7]; and a dual labelled fluorescent TaqMan probe RD-T,5'-(FAM)TAC GTG AGA AAC GCT CAT GAC AGC AAA GTC T(TAMRA)-3' [SEQ ID NO: 8]. Primer/probe combinations were designed using the Primer Express software (Perkin-Elmer, Foster City, CA, U.S.A.). Sequence data for the RhD gene were as previously described (Le Van Kim *et al* 1992).

The beta-globin TaqMan system consisted of the amplification primers beta-globin-354F, 5'-GTG CAC CTG ACT CCT GAG GAG A-3' [SEQ ID NO: 9]; beta-globin-455R, 5'-CCT TGA TAC CAA CCT GCC CAG-3'; and a dual labelled fluorescent TaqMan probe beta-globin-402T, 5'-(FAM)AAG GTG AAC GTG GAT GAA GTT GGT GG(TAMRA)-3' [SEQ ID NO: 10]. Primer/probe combinations were designed using the Primer Express software (Perkin-Elmer, Poster City, CA, U.S.A.). Sequence data were obtained from the GenBank Sequence Database: accession number U01317.

Results

Development of real time TaqMan PCR

The real time sequence detector is able to measure the

5   fluorescence intensity of the liberated reporter molecules cycle after cycle.

A parameter, termed the threshold cycle (C_T), could be defined which was

set at 10 standard deviations above the mean base-line fluorescence

calculated from cycles 1 to 15 (Heid *et al* 1996). An amplification reaction

in which the fluorescence intensity rises above the threshold during the

10   course of thermal cycling is defined as a positive reaction.

To determine the sensitivity of TaqMan PCR, serial dilutions

of genomic DNA isolated from a RhD-positive individual were made in

water consisting of the DNA equivalent from 1,000 cells to 1 cell and

subjected to analysis by the SRY TaqMan system. The fewer the number

15   of target molecules, the more amplification cycles were needed to produce

a certain quantity of reporter molecules. The system was sensitive enough

to detect the DNA equivalent from a single target cell.

Correlation of serology and genotyping of the RhD-negative women

The 21 pregnant women enrolled in this study were all

20   serologically RhD-negative. Genomic DNA (10 ng) isolated from the buffy

coat from each woman was subjected to the RhD TaqMan assay and in

each case a negative result was found; thus demonstrating complete

correlation between the serology and genotyping.

RhD genotyping from DNA isolated from maternal plasma

25   DNA extracted from the plasma of the 21 RhD-negative

pregnant women were subjected to the RhD TaqMan assay. There was

complete correlation between the foetal RhD genotype predicted from

maternal plasma analysis and the result obtained from genotyping the

amniotic fluid and serological testing of the cord blood (Table 1).

19

As a control for the amplifiability of DNA extracted from maternal plasma, these samples were also subjected to the beta-globin TaqMan assay. In every case, a positive TaqMan signal was generated.

5   **Discussion**

In this study we have demonstrated the feasibility of performing non-invasive foetal RhD genotyping from maternal plasma. This represents the first description of single gene diagnosis from maternal plasma. Our results indicate that this form of genotyping is highly accurate

10 and can potentially be used for clinical diagnosis. This high accuracy is probably the result of the high concentration of foetal DNA in maternal plasma.

The rhesus family of polypeptides are encoded by two related genes: the CcEe gene and the RhD gene (Le Van Kim *et al* 1992; Chérif-

15 Zahar *et al* 1990). Due to the complexity of the Rh genetic systems, a number of primer sets have been described for RhD genotyping (Bennet *et al* 1993; Lo *et al* 1993; Aubin *et al* 1997). In order to ensure the accuracy of our genotyping system in the study samples, we performed a control genotyping of buffy coat DNA of our patient population. In all cases there

20 was complete correlation between serology and genotype. It is likely that for robust clinical diagnosis, multiple primer sets are preferred. The TaqMan chemistry can easily accommodate the inclusion of multiple primer/probe sets.

The correlation between the severity of foetal haemolytic

25 disease and maternal and-D level is an area which required further investigation. It is possible that increased amount of foetal DNA is liberated into the maternal circulation in the presence of increased foetal haemolysis.

Table 1

RhDd genotyping from plasma from RhD-negative pregnant women

Case	Foetal RhD genotype	Maternal Plasma RhD TaqMan signal
1	-	-
2	-	-
3	-	-
4	+	+
5	+	+
6	-	-
7	-	-
8	+	+
9	+	+
10	-	-
11	+	+
12	+	+
13	+	+
14	+	+
15	-	-
16	+	+
17	+	+
18	+	+
19	+	+
20	+	+
21	+	+

Example 4

5   Elevation of foetal DNA concentration in maternal serum in pre-eclamptic pregnancies

Introduction

Pre-eclampsia is an important cause of maternal and foetal

10   mortality and morbidity. Despite much research, the pathogenesis of this condition is still unclear. The disorder is mainly recognised by the concurrence of pregnancy-induced changes which regress after delivery, of which hypertension and proteinuria are the most commonly used clinical criteria. Some investigators have suggested that pre-eclampsia is the

result of abnormal trophoblastic implantation, probably mediated by
immunological mechanisms. Other investigators have found pathological
changes in the spiral arteries in the decidua and myometrium in which
partial occlusion by fibrinoid material is one feature.

5   In this Example we use a real time quantitative PCR assay to
show the concentration of foetal DNA in the serum of women suffering from
pre-eclampsia. Y chromosomal sequences from male foetuses were used
as a foetal marker.

Materials and Methods

10   **Patients**

Pregnant women attending the Department of Obstetrics &
Gynaecology at the Prince of Wales Hospital, Shatin, Hong Kong and the
Nuffield Department of Obstetrics & Gynaecology, Oxford, U.K. were
recruited with informed consent. Approval was obtained from the

15   Research Ethics Committee of The Chinese University of Hong Kong and
the Central Oxfordshire Research Ethics Committee. Pre-eclampsia was
defined as a sustained rise in diastolic blood pressure to 90 mmHg or
higher from previously lower values, with new and sustained proteinuria in
the absence of urinary tract infection. The control pregnant women were

20   not on medication and had no hypertension or proteinuria (defined as more
than a trace on dipstick urinalysis). The pre-eclamptic and control subjects
were matched for gestational age.

Sample preparation

Blood samples were collected into plain tubes. Following

25   blood clotting, the samples were centrifuged at 3000 g, and serum were
carefully removed and transferred into plain polypropylene tubes. The
samples were stored at -70 °C or -20 °C until further processing.

DNA extraction from plasma and serum samples

DNA from serum samples were extracted using a QIAamp

30   Blood Kit (Qiagen, Hilden, Germany) using the "blood and body fluid

protocol" as recommended by the manufacturer (Chen *et al* 1996). Four hundred to 800 μl of plasma/serum sample was used for DNA extraction per column. The exact amount used was documented to enable the calculation of target DNA concentration.

5 **Real time quantitative PCR**

Real time quantitative PCR analysis was performed as described in Example 2.

Results

10 **Quantitative analysis of foetal SRY gene from maternal serum**

Real time quantitative SRY PCR was carried out for serum DNA extracted from pre-eclamptic and control patients. Data from individual cases are plotted in Figure 2. The median foetal DNA concentrations in pre-eclamptic and control pregnancies were 381

15 copies/ml and 76 copies/ml, respectively. Foetal DNA concentration was higher in pre-eclamptic than control pregnancies (Mann-Whitney U Test, $p < 0.0001$).

Discussion

20 Our data indicate that the concentration of foetal DNA is higher in pre-eclamptic compared with non-pre-eclamptic pregnancies. These results indicate that foetal DNA concentration measurement in maternal plasma may be used as a new marker for pre-eclampsia. Compared with other markers for pre-eclampsia, foetal DNA measurement

25 is unique in that it is a genetic marker while other markers, such as activin A and inhibin A, are generally hormonal markers. By its nature, a test based on a genetic marker has the advantage that it is completely foetal-specific.

Further research will be required to investigate whether the

30 level of foetal DNA is related to the severity of pre-eclampsia. Our

discovery also opens up research into the potential application of foetal DNA quantitation to predict the occurrence of pre-eclampsia, prior to the development of clinical signs such as hypertension and proteinuria.

5   The mechanism by which increased amounts of foetal DNA is liberated into the circulation of pre-eclamptic women is unclear at present. Possible mechanisms include damage to the placental interface resulting in foetal cell death and the consequent release of foetal DNA into maternal circulation. A second mechanism is due to the increased trafficking of foetal cells into maternal circulation in pre-eclampsia. Foetal DNA is then

10   liberated following their destruction in the maternal circulation. Future studies correlating the levels of foetal cells and foetal DNA would be necessary to address these issues.

Example 5

15   **Quantitative analysis of foetal DNA in maternal plasma and serum**

Introduction

        We have demonstrated that foetal DNA is present in maternal plasma and serum. Detection of foetal DNA sequences was possible in

20   80% and 70% of cases using just 10 μl of boiled plasma and serum, respectively (Lo et al. 1997).

        These observations indicate that maternal plasma/serum DNA may be a useful source of material for the non-invasive prenatal diagnosis of certain genetic disorders. To demonstrate that clinical

25   applications are possible, a number of important questions need to be answered. First, foetal DNA in maternal plasma and serum needs to be shown to be present in sufficient quantities for reliable molecular diagnosis to be carried out. Second, data on the variation of foetal DNA in maternal plasma and serum with regard to gestation age is required to determine

30   the applicability of this technology to early prenatal diagnosis.

24

In this Example we have addressed both of these issues by developing a real time quantitative TaqMan polymerase chain reaction (PCR) assay (Heid *et al.* 1996) for measuring the copy numbers of foetal DNA molecules in maternal plasma and serum. This technique permits

5 continuous optical monitoring of the progress of an amplification reaction, giving accurate target quantitation over a wide concentration range. Our data show that foetal DNA is present in maternal plasma and serum at concentrations similar to those achieved by many foetal cell enrichment protocols. We have also investigated the changes of foetal DNA

10 concentration in maternal serum at different gestational ages. Using this plasma or serum-based approach, we show that the reliable detection of foetal DNA is achievable and therefore useful for the non-invasive prenatal diagnosis of selected genetic disorders.

Subjects and Methods

15 **Patients**

Pregnant women attending the Department of Obstetrics & Gynecology at the Prince of Wales Hospital, Shatin, Hong Kong were recruited with informed consent. Approval was obtained from the Research Ethics Committee of The Chinese University of Hong Kong. For

20 women studied at a single time point, early pregnancy samples were obtained prior to amniocentesis or chorionic villus sampling while late pregnancy samples were collected just prior to delivery. Five to ten ml of maternal peripheral blood was collected each into one tube containing EDTA and one plain tube. Subjects studied at multiple time points were

25 recruited from the *in vitro* fertilization program, prior to conception. Five to ten ml of maternal blood from these subjects was collected into a plain tube at each studied time point. For women undergoing prenatal diagnosis, the sex of the baby was ascertained from cytogenetic results from the amniocentesis or chorionic villus samples. For women recruited

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just prior to delivery or from the *in vitro* fertilization program, foetal sex was noted at the time of delivery.

Sample preparation

5   Blood samples were centrifuged at 3000 g, and plasma and serum were carefully removed from the EDTA-containing and plain tubes, respectively, and transferred into plain polypropylene tubes. Great care was taken to ensure that the buffy coat or the blood clot was undisturbed when plasma or serum samples, respectively, were removed. The plasma and serum samples were recentrifuged at 3000 g and the supernatants

10   were collected into fresh polypropylene tubes. The samples were stored at -20 °C until further processing.

DNA extraction from plasma and serum samples

          DNA from plasma and serum samples were extracted using a QIAamp Blood Kit (Qiagen, Hilden, Germany) using the "blood and body

15   fluid protocol" as recommended by the manufacturer (Chen *et al.* 1996). Four hundred to 800 µl of plasma/serum sample was used for DNA extraction per column. The exact amount used was documented to enable the calculation of target DNA concentration.

Real time quantitative PCR

20   Real time quantitative PCR analysis was performed as described in Example 2, using the SRY TaqMan system and the beta-globin TaqMan system described in the previous Examples.

          Identical thermal profile was used for both the SRY and beta-globin TaqMan systems. Thermal cycling was initiated with a 2-minute

25   incubation at 50°C for the uracil N-glycosylase to act, followed by a first denaturation step of 10 minutes at 95 °C. Then, 40 cycles of 95°C for 15 s and 60°C for 1 minute were carried out.

30

26

Results

Development of real time quantitative PCR

, To determine the dynamic range of real time quantitative
PCR, serial dilutions of male DNA were made in water consisting of the
5   DNA equivalent from 1,000 cells to 1 cell and subjected to analysis by the
SRY TaqMan system. Fig. 3A demonstrates that the amplification curve
shifted to the right as the input target quantity was reduced. This was
expected as reactions with fewer target molecules required more
amplification cycles to produce a certain quantity of reporter molecules
10   than reactions with more target molecules. The system was sensitive
enough to detect the DNA equivalent from a single target cell.

Fig. 3B shows a plot of the threshold cycle (C_T) against the
input target quantity, with the latter plotted on a common log scale. The C_T
was set at 10 standard deviations above the mean base-line fluorescence
15   calculated from cycles 1 to 15 and was proportional to the starting target
copy number used for amplification (Heid *et al.* 1996). The linearity of the
graph demonstrates the large dynamic range and accuracy of real time
quantitative PCR. Similar results were obtained using the beta-globin
TaqMan system (results not shown).

20   The real time quantitative SRY system was insensitive to the
existence of background female DNA from 0 to 12,800 female genome-
equivalents. This greatly simplified the application of this system as within
this range, separate calibration curves did not have to be constructed for
different cases due to the presence of different concentrations of foetal and
25   maternal DNA.

The reproducibility of DNA extraction from plasma and serum
using the Qiagen protocol was tested by performing replicate extractions
(10 for each case) from plasma and serum samples from normal
individuals. These replicate extractions were then subjected to real time

quantitative PCR using the beta-globin system. The coefficient of variation
(CV) of C_T values of these replicate extractions was 1. 1%.

Quantitative analysis using the real time beta-globin TaqMan system

The concentration of beta-globin sequences in maternal
5   plasma and serum samples was used as a measure of the total amount of
extracted DNA, i.e., maternal *and* foetal DNA extracted from plasma and
serum samples from 50 pregnant women was analysed using the beta-
globin TaqMan system. Twenty-five cases were recruited during the first
and second trimesters (gestational age: 11 to 17 weeks) and were denoted
10   as early pregnancy samples in Table 2. The other twenty-five cases were
recruited just prior to delivery (gestational age: 37 to 43 weeks) and were
denoted as late pregnancy samples in Table 1. The concentrations of
beta-globin sequences in maternal plasma and serum are listed in Table 2.
These results show that serum contains more DNA than plasma (Wilcoxon
15   Signed Rank Test, p<O.0005), with a mean concentration of serum DNA
14.6 times that of plasma DNA in our studied population. The
concentration of beta-globin sequences in maternal plasma from early and
late pregnancy samples are compared in Table 2. These data show that
the total amount of plasma DNA increases as pregnancy progresses
20   (Mann-Whitney Rank Sum Test, p<O.0005).

**Quantitative analysis of foetal SRY gene from maternal plasma and
serum**

Real time quantitative analysis using the SRY TaqMan
system was carried out on DNA extracted from maternal plasma and
25   serum to determine the amount of foetal DNA. Of the 25 early pregnancy
samples (gestational age: 11 to 17 weeks), 13 were from women bearing
male foetuses and 12 were from women bearing female foetuses. Of the
25 late pregnancy samples (gestational age: 37 to 43 weeks), 14 were
from women bearing male foetuses and 11 were from women bearing
30   female foetuses. A positive signal was obtained in each of the 27 women

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bearing male foetuses and no signal was detected in each of the 23
women bearing female foetuses. Fourteen women had a history of
delivering a previous male baby and 5 of these were carrying a female
baby in the current studied pregnancy.

5   Quantitative SRY data from the 27 women bearing male
foetuses are summarised in Table 3. These data show that the
concentrations of foetal DNA in plasma and serum are higher in late
gestation than in early gestation (Mann-Whitney Rank Sum Test,
$p < 0.0005$). The mean concentrations of foetal DNA in maternal plasma

10   and serum are 11.5 times and 11.9 times, respectively, higher in late
gestation compared with early gestation. The absolute concentrations of
foetal DNA in maternal plasma and serum were similar in individual cases.
The fractional concentration of foetal DNA in early pregnancy ranges from
0.39% to 11.9% (mean: 3.4%) in plasma and 0.014% to 0.54% (mean:

15   0.13%) in serum. In late pregnancy, the fraction of foetal DNA ranges from
2.33% to 11.4% (mean: 6.2%) in plasma and 0.032% to 3.97% (mean:
1.0%) in serum.

Sequential follow up of women who conceived by *in vitro* fertilization

        Twenty women who conceived by *in vitro* fertilization (IVF)

20   were followed up at pre-conception and at multiple time points during
pregnancy. All twenty subjects had singleton pregnancies as determined
by ultrasound scanning. Twelve women delivered male babies and the
remaining 8 delivered female babies. None of the women carrying male
foetuses had a history of pregnancy-associated complications. Subject S-

25   51 (fig. 4) underwent chorionic villus sampling at 12 weeks. Subjects S-1
and S-56 (fig. 4) had amniocentesis at 16 and 17 weeks, respectively. A
total of 163 serum samples from these 20 women were analysed using the
real time quantitative SRY TaqMan system. None of the 65 serum
samples from the 8 women bearing female babies gave a positive SRY

signal. The concentrations of foetal DNA in the 98 serum samples from women carrying male babies are plotted in fig. 4.

Discussion

5　　　　　　We have developed an accurate real time quantitative PCR system for determining the concentration of foetal DNA in maternal plasma and serum. This system has a number of advantages: (1) a large dynamic range of over 5 orders of magnitude (Heid et al. 1996); (2) a high throughput and fast tumaround time - 96 samples could be simultaneously

10　amplified and quantified in approximately 2 hours; and (3) the use of a homogeneous amplification/detection system which requires no post-PCR processing and therefore minimizes the risk of carryover contamination.

　　　　　　The most important observation in this study is the very high concentration of foetal DNA in maternal plasma and serum. Bianchi et al

15　reported that the average number of foetal cells in maternal blood in normal pregnancies was 19 in 16 ml of maternal blood, i.e., 1.2 cells/ml during the second trimester (Bianchi et al. 1997). Therefore, the mean concentration of foetal DNA in maternal plasma and serum is 21.2 (25.4/1.2) and 23.9 (28.7/1.2) times, respectively, higher than that in the

20　cellular fraction of maternal blood at the same gestation. The relative concentration of foetal to total plasma DNA is even higher. Thus, in early pregnancy, foetal DNA in maternal plasma constitutes a mean of 3.4% of the total plasma DNA. The respective figure in late pregnancy is 6.2%. Hamada et al reported that the frequency of foetal cells in the second

25　trimester was 0.0035% while that in the third trimester was 0.008% (Hamada et al. 1993). The fetomaternal ratio is, therefore, 97Sfold and 775-fold higher in maternal plasma than in the cellular fraction at the respective gestational age. Indeed, the fetomaternal ratio in plasma DNA is comparable to that following many foetal cell enrichment protocols. For

30　example, Bianchi et al reported that following foetal nucleated red cell

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enrichment using fluorescence activated cell sorting, the resulting foetal cells constituted 0.001%-5% of the sorted cell populations as determined by quantitative PCR analysis (Bianchi *et al.* 1994). In a similar study using cell sorting and foetal cell detection using fluorescence in situ hybridization,

5 Sohda *et al* found that on average 4.6% of the sorted cells were of foetal origin (Sohda *et al.* 1997). Maternal plasma, therefore, offers an easily accessible foetal DNA source for prenatal genetic analysis.

We have demonstrated that the absolute concentration of foetal DNA in maternal plasma is similar to that in maternal serum. The

10 main difference lies in the presence of a larger quantity of background maternal DNA in serum compared with plasma, possibly due to the liberation of DNA during the clotting process. While this exerts no noticeable effect on the efficiency of foetal DNA detection using the real time TaqMan system, it is possible that with the use of less sensitive

15 methods, e.g., conventional PCR followed by ethidium stained agarose gel electrophoresis, maternal plasma may be preferable to maternal serum for robust foetal DNA detection.

The high concentration of foetal DNA in maternal plasma and serum has allowed us to reliably detect the presence of foetal genetic

20 material. Of the 263 serum or plasma samples analysed in this study, we were able to detect foetal SRY gene in maternal plasma or serum from every subject who was carrying a male baby at the time of venesection. This robust detection rate was obtained using DNA extracted from just 40-80 µl of maternal plasma and serum. This volume represents a 4-8 fold

25 increase over the 10 µl of boiled maternal plasma or serum reported in our previous study (Lo *et al.* 1997) and results in significant improvement in sensitivity. The specificity was preserved as we did not observe amplification signals from samples obtained pre-conception or from subjects carrying a female foetus. From the data obtained thus far,

30 plasma/serum analysis did not appear to be significantly affected by the

31

persistence of foetal cells from previous pregnancies (Bianchi *et al.* 1996).
Thus, we did not obtain any false positive results from women who had
carried a previous male baby but who were carrying a female baby at the
time of blood sampling for this study.

5   The sequential study on patients undergoing IVF gave a
number of important results. First, all of the 12 patients carrying male
babies were shown to be negative for SRY sequences in their sera prior to
conception. This provided convincing evidence that the SRY sequence
detected by the TaqMan assay did indeed originate from the male foetus in
10   the *current* pregnancy. Second, we were able to detect foetal SRY
sequences as early as the 7th week of gestation; thus indicating that foetal
genetic analysis in maternal plasma/serum could be used in the first
trimester. Third, we showed that foetal DNA concentration increased as
pregnancy progressed (fig 4). This last point was also confirmed by data
15   obtained from women studied at a single time point. Women recruited late
in pregnancy had higher foetal DNA concentrations in their plasma and
serum (Table 3).

          In addition to the increase in foetal DNA concentration as
pregnancy progresses, our data also indicate that maternal plasma DNA
20   also increases with gestation (Table 2). The biologic basis for this
phenomenon is unclear at present. Possible explanations include the
increase in size of the fetomaternal interface as gestation progresses and
possible reduction in DNA clearance associated with other physiologic
changes in pregnancy.

25   For selected disorders, foetal genetic information could be
acquired more economically and rapidly from maternal plasma or serum
than by using foetal cells isolated from maternal blood. We envisage that
foetal DNA analysis in maternal plasma and serum would be most useful in
situations where the determination of foetal-derived paternally-inherited
30   polymorphisms/mutations or genes would be helpful in clinical prenatal

diagnosis (Lo *et al.* 1994). Examples include foetal sex determination for the prenatal diagnosis of sex-linked disorders, foetal rhesus D status determination in sensitized rhesus negative pregnant women (Lo *et al.* 1993), autosomal dominant disorders in which the father carries the

5   mutation and autosomal recessive genetic disorders in which the father and mother carry different mutations (Lo *et al.* 1994), e.g., certain hemoglobinopathies (Camaschella *et al.* 1990) and cystic fibrosis. Due to the much reduced maternal background and high foetal DNA concentration in maternal plasma and serum, we predict that this type of analysis would

10   be much more robust compared with their application for detecting unsorted foetal cells in maternal blood. The ability for allelic discrimination (Lee *et al.* 1993; Livak *et al.* 1995) allows the homogeneous TaqMan assay to be used for this purpose. The high throughput and anti-contamination capability of this system makes it an attractive candidate for

15   large scale clinical application.

        Bianchi *et al* recently reported that foetal cells in maternal blood were increased in aneuploid pregnancies (Bianchi *et al.* 1997) and it has been demonstrated (Example 2) that the foetal DNA concentration in maternal plasma and serum is also elevated in these pregnancies. This

20   provides a new screening test for foetal chromosomal disorders. For this application, foetal DNA quantitation systems can be developed for polymorphic markers outside the Y chromosome so that quantitation can be applied to female foetuses. Autosomal polymorphic systems which may be used for this purpose have already been described (Lo *et al.* 1996).

25   However, foetal cell isolation techniques would still be necessary for a definitive cytogenetic diagnosis. Similarly, foetal cell isolation would also be required for direct mutational analysis of autosomal recessive disorders caused by a single mutation. It is likely that foetal cell isolation and analysis of foetal DNA in maternal plasma/serum would be used as

30   complementary techniques for non-invasive prenatal diagnosis.

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The biologic basis by which foetal DNA is liberated into maternal plasma remains to be elucidated. It is possible that foetal DNA is released from cell lysis resulting from physical and immunologic damage, or through developmentally associated apoptosis of foetal tissues. It is

5   also likely that increased amounts of foetal DNA may be found in conditions associated with placental damage, such as pre-eclampsia. The real time quantitative PCR system described here offers a powerful tool to study these unexplored pathophysiologic aspects of foetal DNA in maternal plasma and may improve our understanding of the fetomaternal

10   relationship.

Table 2

Quantitative analysis of maternal plasma and serum using the beta-globin TaqMan assay

	Mean (copies/ml)	Median (copies/ml)	Range (copies/ml)
Plasma (Early + Late Pregnancy)	3466	1594	356-31875
Serum (Early + Late Pregnancy)	50651	34688	5813-243750
Plasma (Early Pregnancy)	986	975	356-1856
Plasma (Late Pregnancy)	5945	4313	1125-31875

Table 3

Quantitation of foetal DNA in maternal plasma and serum: relationship with gestational age

SRY concentation (copies/ml)

	Early Pregnancy		Late Pregnancy	
	Plasma	Serum	Plasma	Serum
Range	3.3 - 69.4	4.0- 58.1	76.9 - 769	33.8 - 900
Mean	25.4	28.7	292.2	342.1
Median	20.6	19.5	244.0	286.0

Figure Legends

Figure 1. Foetal DNA in maternal serum from women carrying aneuploid and normal foetuses. The control and aneuploid groups are as

5   indicated on the x-axis. The foetal SRY DNA concentrations expressed in copies/ml are plotted on the y-axis.

Figure 2. Foetal DNA in maternal serum in pre-eclamptic and non-pre-eclamptic pregnancies. The pre-eclamptic and control groups are as indicated on the x-axis. The foetal SRY DNA concentrations expressed

10   in copies/ml are plotted on the y-axis.

Figure 3. Real time quantitative PCR.

A, Amplification plots obtained using real time quantitative PCR for the SRY gene. Each plot corresponds to a particular input target quantity marked by a corresponding symbol. The x-axis denotes the cycle number

15   of a quantitative PCR reaction. The y-axis denotes the ΔRn which is the fluorescence intensity over the background (Heid *et al.* 1996).

B, Plot of the threshold cycle (C_T) against the input target quantity (common log scale). The correlation coefficient is 0.986.

Figure 4. Sequential study of 12 women bearing male

20   fetuses who conceived by *in vitro* fertilization. Each case is denoted by a unique recruitment case number. The x-axis denotes the gestation at which the serum sample was obtained. A gestation age of zero denotes the pre-conception sample. The y-axis denotes the concentration of foetal SRY in maternal serum expressed in copies/ml. The scale has been

25   optimized for the concentration range for each case.

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CLAIMS:

1.   A detection method performed on a maternal serum or plasma sample from a pregnant female, which method comprises detecting the presence of a nucleic acid of foetal origin in the sample.

5   2.   The method according to claim 1, comprising amplifying the foetal nucleic acid to enable detection.

3.   The method according to claim 2, wherein the foetal nucleic acid is amplified by the polymerase chain reaction.

4.   The method according to claim 2 or claim 3, wherein at least

10   one foetal sequence specific oligonucleotide primer is used in the amplification.

5.   The method according to any one of claims 1 to 4, wherein the foetal nucleic acid is detected by means of a sequence specific probe.

6.   The method according to any one of claims 1 to 5, wherein

15   the presence of a foetal nucleic acid sequence from the Y chromosome is detected.

7.   The method according to claim 6, wherein the Y chromosome sequence is from the DYS14 locus.

8.   The method according to claim 6, wherein the Y chromosome

20   sequence is from the SRY gene.

9.   The method according to any one of claims 1 to 5, wherein the presence of a foetal nucleic acid from a paternally-inherited non-Y chromosome is detected.

10.   The method according to claim 9, wherein the non-Y

25   sequence is a blood group antigen gene such as the Rhesus D gene.

11.   The method according to claim 9, wherein the non-Y sequence is a gene which confers a disease phenotype in the foetus, such as the Rhesus D gene.

12.   The method according to claim 10 or claim 11, for Rhesus D

30   genotyping a foetus in a Rhesus D negative mother.

40

13.   The method according to any one of claims 6 to 8, for
determining the sex of the foetus.

14.   The method according to any one of claims 6 to 12, which
comprises determining the concentration of the foetal nucleic acid
sequence in the maternal serum or plasma.

15.   The method according to claim 14, wherein the determination
of the concentration of foetal nucleic acid sequence in the maternal serum
or plasma is by quantitative PCR.

16.   The method according to claim 14 or claim 15, for the
detection of a maternal or foetal condition in which the level of foetal DNA
in the maternal serum or plasma is higher or lower than normal.

17   The method according to any one of claims 14 to 16, wherein
the pattern of variation of foetal DNA concentration in the maternal serum
or plasma at particular stages of gestation is different from normal.

18.   The method according to claim 16 or claim 17, for detection
of pre-eclampsia.

19.   The method according to claim 16 or claim 17, for detection
of a foetal chromosomal aneuploidy.

20.   The method according to any one of claims 1 to 19, wherein
the sample contains foetal DNA at a fractional concentration of total DNA
of at least about 0.14%, without subjecting it to a foetal DNA enrichment
step.

21.   The method according to claim 20, wherein the fractional
concentration of foetal DNA is at least about 0.39%.

22.   A method of performing a prenatal diagnosis, which method
comprises the steps of:

(i)   providing a maternal blood sample;

(ii)   separating the sample into a cellular and a non-cellular
fraction;

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(iii)   detecting the presence of a nucleic acid of foetal origin in the
non-cellular fraction according to the method of any one of
claims 1 to 21;

(iv)   providing a diagnosis based on the presence and/or quantity
5   and/or sequence of the foetal nucleic acid.

23.   The method according to claim 22, wherein the non-cellular
fraction as used in step (iii) is a plasma fraction.

24.   The method according to claim 22, including performing the
further step of allowing clotting in the maternal sample and using the
10   resulting serum in step (iii).

25.   A method of performing a prenatal diagnosis on a maternal
blood sample, which method comprises removing all or substantially all
nucleated and anucleated cell populations from the blood sample and
subjecting the remaining fluid to a test for foetal nucleic acid indicative of a
15   maternal or foetal condition or characteristic.

26.   A method of performing a prenatal diagnosis on a maternal
blood sample, which method comprises obtaining a non-cellular fraction of
the blood sample and performing nucleic acid analysis on the fraction.
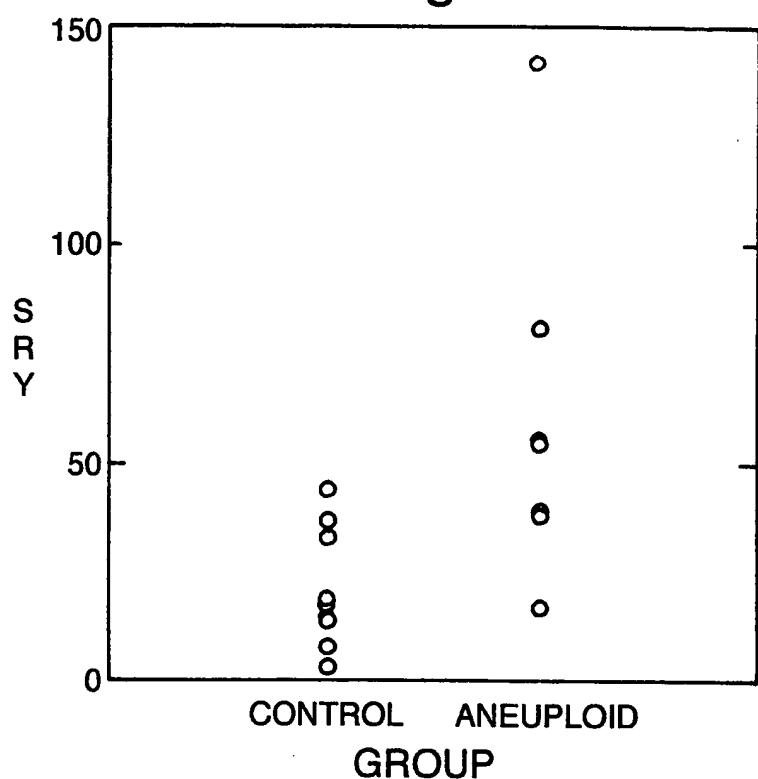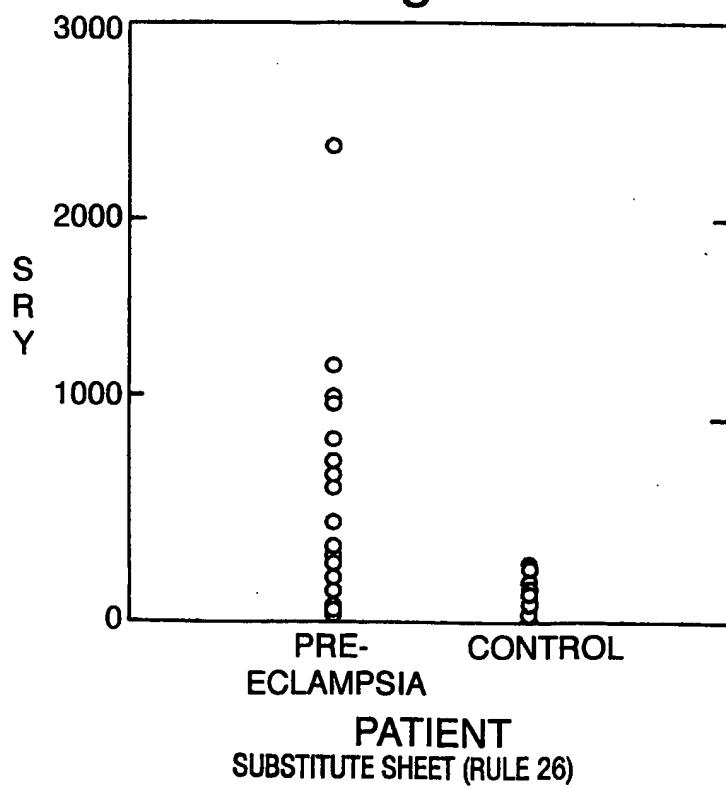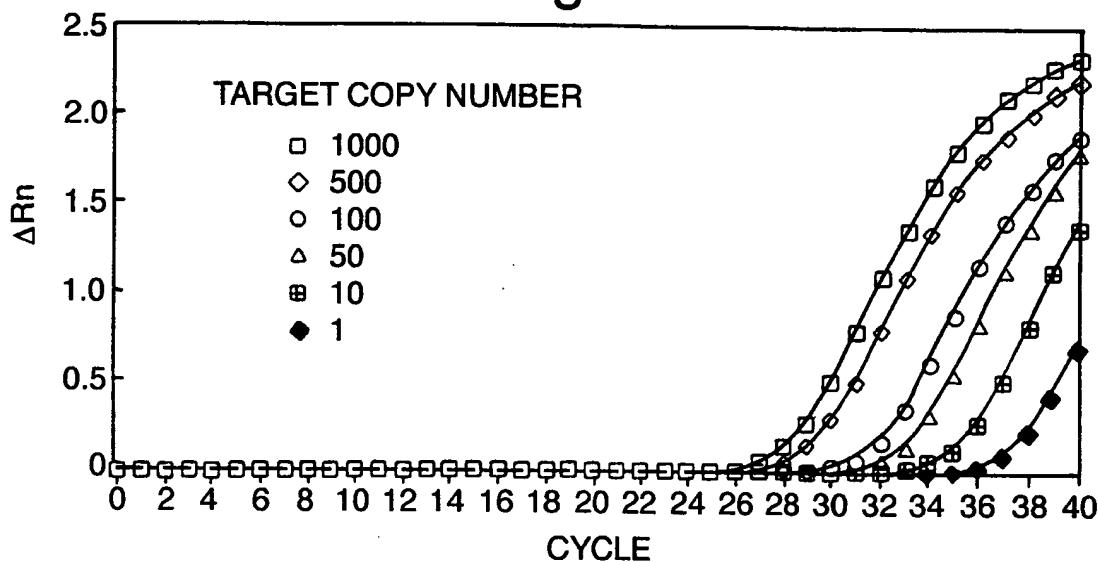
Fig.1.

Fig.2.

Fig.3A.

Fig.3B.
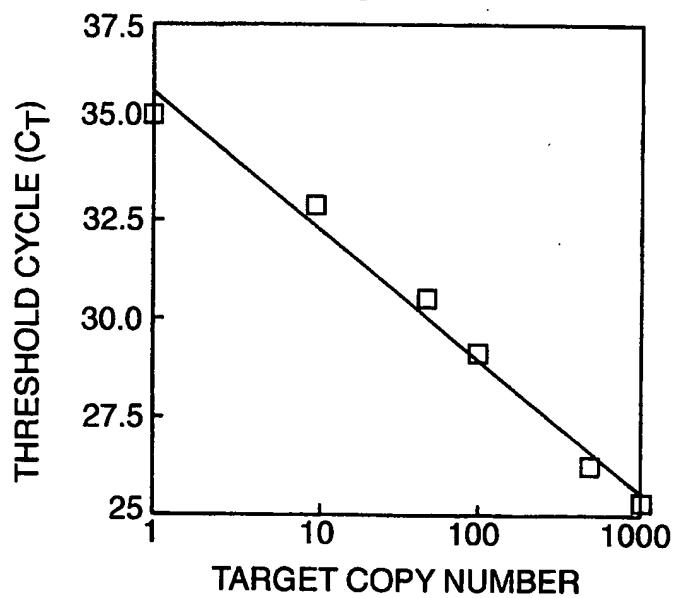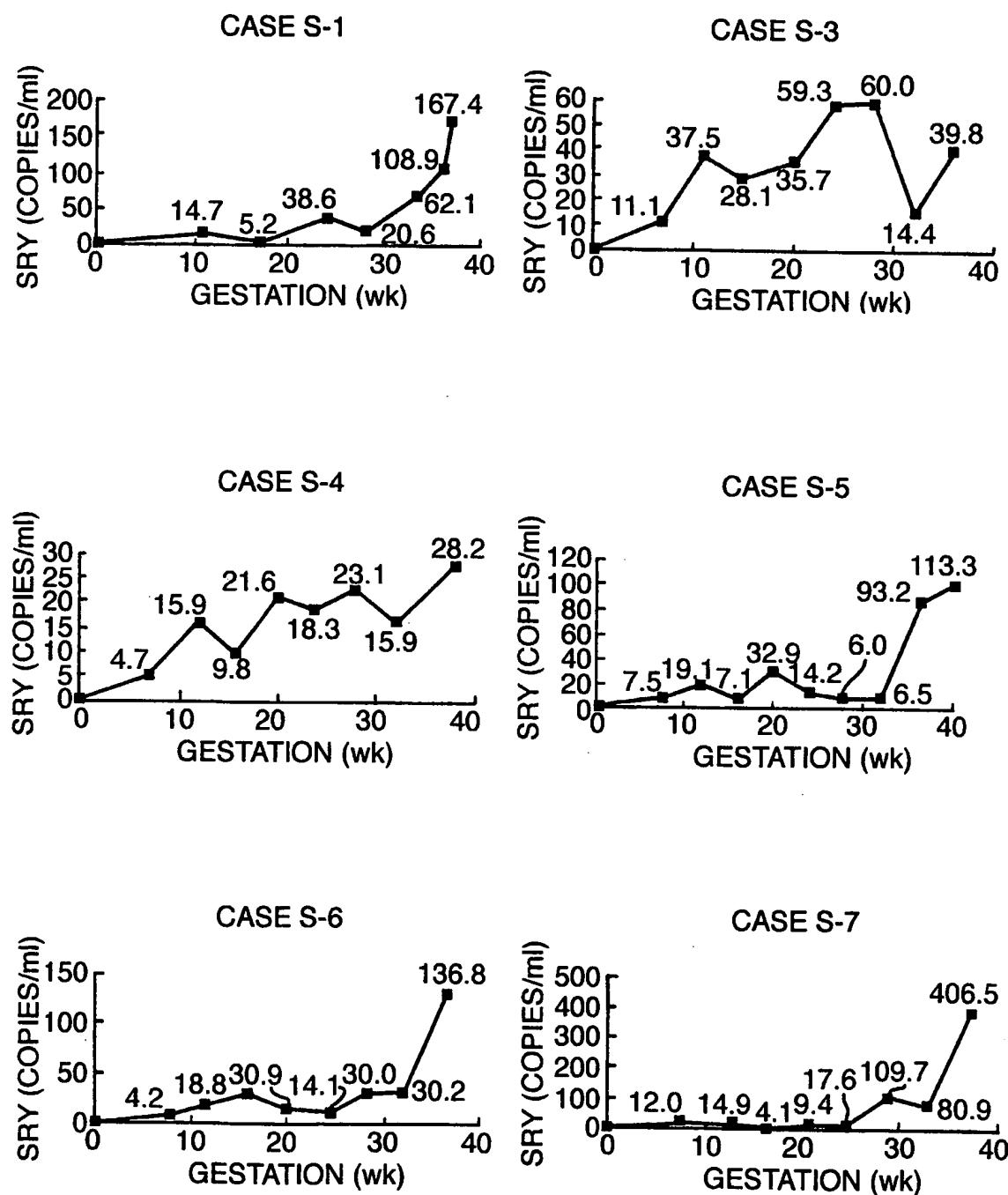
Fig.4.

CASE S-1

CASE S-3

CASE S-4

CASE S-5

CASE S-6

CASE S-7

Fig.4(Cont.)

CASE S-15

CASE S-17

CASE S-51

CASE S-52

CASE S-56

CASE S-65
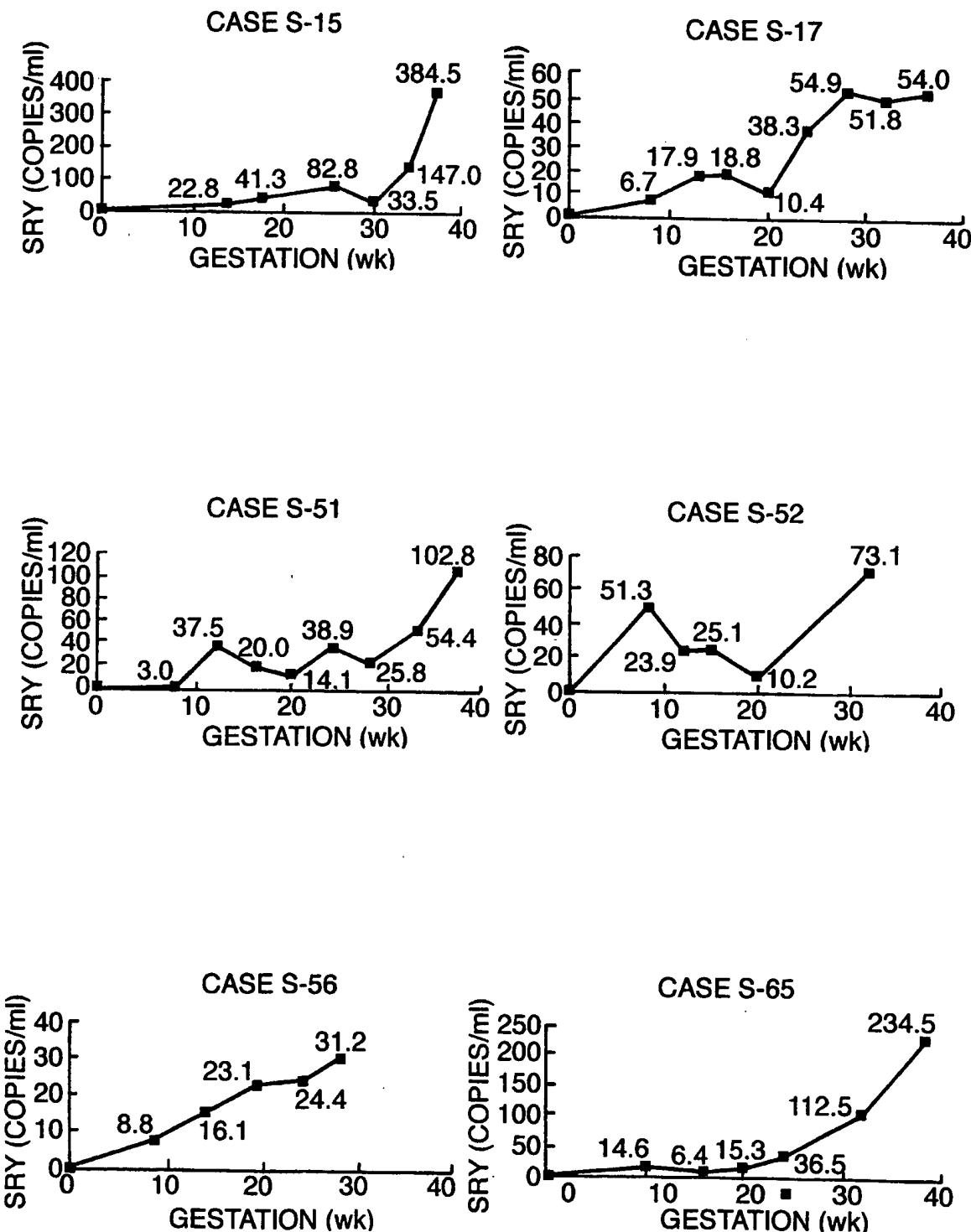
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6   C12Q1/68   G01N33/53

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6   C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 91 08304 A (ISIS INNOVATION) 13 June 1991 cited in the application see abstract; claims	1-3,6,13
A	GB 2 299 166 A (ANKER PHILIPPE ;STROUN MAURICE (CH); VASIOUKHIN VALERI (US)) 25 September 1996 cited in the application see abstract; claims	1-3
A	WO 95 06137 A (AUSTRALIAN RED CROSS ;QUEENSLAND INST MED RES (AU); HYLAND CATHERI) 2 March 1995 see abstract; claims	1-3,10, 11

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[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

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Date of the actual completion of theinternational search	Date of mailing of the international search report
3 July 1998	21/07/1998

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	FOWKE K R ET AL: "Genetic analysis of human DNA recovered from minute amounts of serum or plasma" JOURNAL OF IMMUNOLOGICAL METHODS, vol. 180, no. 1, 13 March 1995, page 45-51 XP004021069 see abstract	1-3
P,X	DATABASE MEDLINE US NATIONAL LIBRARY OF MEDICINE (NLM), BETHESDA, MD, US AN (NLM) 97420079, LO YM ET AL: "Presence of fetal DNA in maternal plasma and serum." XP002070361 cited in the application see abstract & LANCET, AUG 16 1997, 350 (9076) P485-7, ENGLAND,	1-3,6, 13,22-26

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Patent document cited in search report			Publication date	Patent family member(s)			Publication date
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GB 2299166	A		25-09-1996	CH	686982	A	15-08-1996
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